Biochemical and Biophysical Research Communications **270,** 1036–1040 (2000)
doi:10.1006/bbrc.2000.2566, available online at http://www.idealibrary.com on IDEAL®

Stimulation of Yeast 3-Phosphoglycerate Kinase Gene Promoter by Paraquat

Neville Vassallo, Dolores R. Galea, William H. Bannister, and Rena Balzan[1]
Department of Physiology and Biochemistry, University of Malta, Msida, MSD 06, Malta

Received March 24, 2000

Yeast cells exposed to adverse conditions employ a number of defense mechanisms in order to respond effectively to the stress and sustain a high proliferation rate. It has been shown that several glycolytic enzymes are induced upon heat treatment of yeast. In this work, we used a reporter plasmid construct to study the effects of oxidative stress, induced by the $O_2^{\bullet-}$-generating compound paraquat (PQ), on the yeast 3-phosphoglycerate kinase gene (*PGK*) promoter. Our results show that (i) moderate, as opposed to excessive, doses of PQ induce increased stimulation of the *PGK* promoter, at midlogarithmic phase of growth; and (ii) the thiol antioxidant *N*-acetylcysteine cancels this stimulatory effect. These observations may represent one aspect of a more general role for glycolysis in maintaining the energy pools of yeast cells under stress.

***Key Words:* Saccharomyces cerevisiae; 3-phosphoglycerate kinase gene promoter; glycolysis; oxidative stress response.**

The unicellular eukaryotic organism *Saccharomyces cerevisiae* has been extensively used as a model experimental system for the study of oxidative stress responses (1). Oxidative stress can be induced using a variety of redox cycling compounds that can act intracellulary to generate the superoxide radical ($O_2^{\bullet-}$), hydroxyl ($HO^{\bullet}$) radical, and hydrogen peroxide (H_2O_2). One such compound is paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride; PQ) which generates $O_2^{\bullet-}$ (2). While investigating the response of yeast cells to oxidative stress induced by PQ, we have recently observed a possible stimulatory effect of PQ on the promoter of the glycolytic gene 3-phosphoglycerate kinase (*PGK*) in plasmid constructs of the promoter and the *Escherichia coli* iron superoxide dismutase (FeSOD) gene (3). The PGK enzyme is highly expressed in *S. cerevisiae,* contributing to approximately 5% of the total cellular protein (4). In view of the knowledge that oxidative stress can compromise a cell's ability to generate ATP (5), particularly by interfering with normal mitochondrial energy metabolism, we wanted to examine whether generation of reactive oxygen species (ROS), by paraquat, stimulates the *PGK* promoter in yeast. Using a reporter gene construct with the yeast *PGK* promoter sequence, we show that sublethal, as opposed to lethal, doses of PQ result in increased stimulation of the *PGK* promoter at midlogarithmic phase of growth. Addition of the antioxidant *N*-acetylcysteine (NAC) abolishes this stimulatory effect. Furthermore, brief (1 h) exposure to PQ does not elicit increased transcription from the *PGK* promoter, indicating that the biological function of this glycolytic response is probably not intended for immediate protection against stress.

Abbreviations used: PQ, paraquat; PGK, 3-phosphoglycerate kinase; ROS, reactive oxygen species; NAC, *N*-acetylcysteine.

[1] To whom correspondence should be addressed. Fax: +356-310577. E-mail: rbal1@um.edu.mt.

MATERIALS AND METHODS

Bacterial strain and culture conditions. The *E. coli* strain used in the standard cloning procedures was XL1-Blue [*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac-F′ proAB*$^+$ *lacI*^{q} *lacZΔM15 Tn10(Tet′)*] obtained from Stratagene. The media used were (i) 2TY (Bacto-Tryptone, 1.6%; Bacto yeast extract, 1%; NaCl, 0.5%), and (ii) LB medium (Bacto-Tryptone, 1%; Bacto yeast extract, 0.5%; NaCl, 1%). Ampicillin and tetracycline were added at 100 μg/ml and 50 μg/ml, respectively, when required. For plates, 2% agar was used and incubation was at 37°C. Liquid cultures were maintained aerobically at 37°C on a controlled environmental incubator shaker (New Brunswick Scientific) at 300 rpm.

Yeast strain and culture conditions. The *S. cerevisiae* strain used was EG103 (DBY746; MATα *leu2-3,112 his3Δ1 trp1-289a ura3-52 GAL*$^+$), kindly provided by Edith Gralla of UCLA. The culture media used were (i) YEPD (Bacto yeast extract, 1%; Bacto-peptone, 2%; glucose, 2%), and (ii) minimal medium (Bacto yeast nitrogen base without amino acids, 0.67%; glucose, 2%; L-histidine–HCl, 100 μg/ml; L-lysine–HCl, 30 μg/ml; L-tryptophan, 40 μg/ml; L-methionine 20 μg/ml; adenine sulfate 40 μg/ml; uracil 25 μg/ml; L-leucine 90 μg/ml, as required). For plates, 2% agar was used and incubation was at 28°C. X-gal plates were made as described in (6). Aerobic growth in liquid culture was maintained at 28°C with constant shaking at 300 rpm.

Construction of plasmid YEp363PZ. YEp363PZ was constructed from plasmid YEp363 (7), kindly supplied by A. Tzagoloff of Columbia University, by cloning the yeast *PGK* promoter (−1030 to +8) in

0006-291X/00 $35.00

sequence with *E. coli lacZ*. The *PGK* promoter was excised from YEp/PGK-F (8) by digestion with *Asu*II, while YEp363 was linearized at the multiple cloning site upstream of the *E. coli lacZ* using *Pst*I. Both fragments were blunt-ended using Klenow enzyme, and subsequently ligated. The ligation was such that the inserted *PGK* promoter was directing transcription of the *lacZ* gene. All molecular cloning experiments were carried out as specified by Sambrook *et al.* (9). The plasmid construct sequence was confirmed by DNA sequencing using the dideoxy method (10) and Sequenase I enzyme (United States Biochemical).

Expression of E. coli β-galactosidase gene, under the control of the PGK promoter, in yeast cells. *S. cerevisiae* EG103 cells were transformed with the plasmid vector YEp363PZ by the lithium acetate method (11), producing the strain EG103PZ. To demonstrate β-galactosidase (β-gal) expression, EG103PZ cells were plated onto synthetic complete medium lacking leucine (SC-Leu) and containing 20 mg/ml X-gal (Boehringer Mannheim). Following an incubation of 36 h, characteristic blue colonies of yeast cells expressing β-galactosidase were visible.

Exposure to paraquat and preparation of protein extract. In the growth curve experiments, the starting cell density was 1.5×10^6 cells/ml. Paraquat (Sigma) was then added to the desired concentration (1 mM or 3 mM). Cell growth was monitored by measuring the optical density at 600 nm in a Perkin–Elmer Lambda 17 spectrophotometer, after appropriate dilution of the cell culture samples. At each designated phase of growth, three 5-ml samples were retrieved from the large culture for total cell protein extract preparation, followed by β-gal assays on the extract.

In the 1-h exposure experiments, EG103PZ cells were grown in 100 ml of SC-leu medium to early logarithmic phase of growth (OD_{600} = 0.6). The culture was then divided into 5-ml aliquots and sets of three aliquots were exposed to a different PQ concentration or to mild heat shock (38°C). During the 1-h exposure, the control set and those being exposed to PQ were kept at 28°C in a shaking incubator. At the end of the exposure, total cell protein extracts were prepared from each 5 ml culture, followed by β-gal assays on the extract. Total cellular protein extracts were prepared according to Rose *et al.* (6).

Determination of plasmid copy number. Cultures were grown in the absence and presence of 1 mM paraquat, in the same conditions as described above, and cells were harvested at midlog phase. Total yeast DNA was prepared using the method described by Rose *et al.* (6). Approximately 15 μg of total DNA were digested overnight with *Hin*dIII, and the digest run on a 0.7% agarose gel. The most prominent bands seen were those due to the plasmid restriction fragments (*Hin*dIII linearizes the plasmid YEp363PZ to produce a 9.4 kb fragment) and those due to the restriction fragments of ribosomal DNA (*Hin*dIII generates a 6.4 kb repeat fragment from rDNA). The gels were scanned using a Bio-Rad Fluor-S Multimager, and the plasmid copy number in each case was determined using the formula: copy number = ((area under plasmid peak) × (length of rDNA fragment)/(area under rDNA peak) × (length of plasmid fragment)) × 100 (12).

Miscellaneous. Protein concentration was measured using the Bio-Rad assay according to the manufacturer's instructions. Functional β-gal assays were performed as described by Rose *et al.* (6), using the substrate o-nitrophenyl-β-D-galactoside (ONPG) (Boehringer Mannheim). Activity was normalized to the protein concentration of the total cell extract. β-gal ELISA (Boehringer Mannheim) was carried out as described by the manufacturer.

RESULTS

Growth of S. cerevisiae in the presence of 1 mM paraquat results in increased stimulation of the PGK promoter at midlogarithmic phase of growth. It has been previously shown that when yeast cells deficient in copper, zinc superoxide dismutase (Cu,ZnSOD), and carrying either a multicopy or a centromeric plasmid encoding the *E. coli* FeSOD gene, under the transcriptional control of the yeast *PGK* promoter, were exposed to 1 mM PQ, FeSOD activity was increased almost two- and sixfold, respectively (3). To further investigate the response of this glycolytic promoter to oxidative stress generated by PQ, we transformed EG103 cells with plasmid YEp363PZ, containing the yeast *PGK* promoter fused to the bacterial *lacZ* gene, which codes for β-galactosidase. The recombinant EG103PZ cells were then exposed to PQ as described in the experiments below.

EG103PZ cells were grown from early-log phase in the presence of 1 mM PQ. At midlog (points M0 and M1 in Fig. 1A) and early stationary (points S0 and S1 in Fig. 1A) phases, the β-gal activity of the cells exposed to PQ and of the control cells was determined. The results showed a 50% increase in β-gal activity at midlog phase, as a result of growth in the presence of 1 mM PQ. On the other hand, assays from extracts sampled at stationary phase revealed no stimulatory effect of PQ exposure on β-gal levels (Fig. 1B(i)).

Since the reporter gene was being expressed from a multicopy plasmid, the possibility arose that the data showing increased β-gal activity from the 1 mM PQ-exposed culture might be reflecting an unknown selection of PQ for cells containing the higher number of plasmids. We therefore carried out a comparison of plasmid copy number between cells exposed to PQ and control cells, both sampled at midlog phase. The results, shown in Figs. 2A and 2B, demonstrate a similar number of plasmids from yeast cultured in the presence and in the absence of PQ.

To further confirm activation of the *PGK* promoter by PQ, we repeated the above experiments using β-gal ELISA to measure the actual amount of β-gal protein produced. The results are illustrated in Fig. 1B(ii), and verify induction of the *PGK* promoter by PQ at midlog phase.

Growth in the presence of higher, lethal concentrations of paraquat is not associated with induction of the PGK promoter. Evaluation of the cytotoxicity of 1 and 3 mM PQ on yeast using growth curves, showed that 3 mM PQ resulted in a marked inhibition of cell growth and increased cell death (Fig. 1A). Therefore, functional β-gal assays were performed at midlog and stationary phase, as in the previous experiments, on cells growing in the presence of 3 mM PQ (points M3 and S3 in Fig. 1A). No induction of the *PGK* promoter was evident from these results (Fig. 1B(i)).

The antioxidant N-acetylcysteine results in loss of the stimulatory effect of 1 mM PQ on the PGK promoter. Growth curve experiments indicated that 4 mM NAC abolishes the growth inhibitory effect of 1 mM PQ on EG103PZ cells (data not shown). We therefore tested

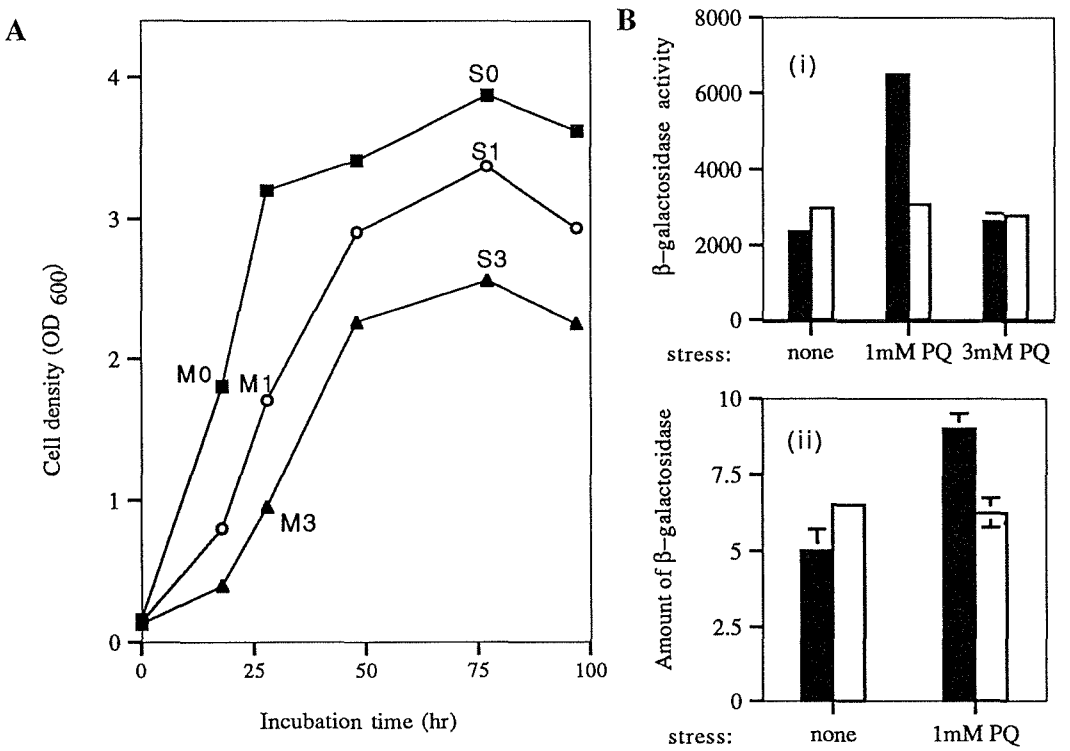

FIG. 1. Exposure of *S. cerevisiae* cells to paraquat and its effect on the *PGK* promoter. EG103PZ cells were grown in SC-Leu medium containing 0 mM PQ (■), 1 mM PQ (○), or 3 mM PQ (▲). At midlog (points M0, M1, and M3 in Fig. 1A) and early stationary phase (points S0, S1, and S3 in Fig. 1A) a set of three 5-ml aliquots were harvested from each culture, extracts prepared and immediately assayed for β-gal activity (Fig. 1B(i)), or for β-gal quantification by ELISA (Fig. 1B(ii)), as described under Materials and Methods. Filled bars represent values from midlog phase cultures; white bars represent values from stationary phase cultures. All values shown are the means of at least three independent determinations ± SD. Specific activity of β-gal is quoted as nmol ONPG hydrolyzed/min/mg total protein. Quantification of β-gal is quoted as μg of β-gal/mg total protein. Error bars are ±1 SD and appear where sufficiently large.

whether NAC could also, by virtue of its antioxidant properties, annul *PGK* promoter stimulation by PQ. A set of three EG103PZ 5-ml cultures, each in triplicate, were prepared in SC-Leu medium: 1 mM PQ was added to two of the culture sets upon starting aerobic incubation. When the cells had grown to midlog phase, 4 mM NAC was added to one of the culture sets growing in the presence of 1 mM PQ. After 2 h all cultures were harvested and assayed for β-gal activity. As shown in Fig. 3, we found that adding the antioxidant NAC results in loss of the increased β-gal activity levels obtained from the cultures that had been continually exposed to 1 mM PQ.

One-hour exposure to paraquat does not elicit induction of the PGK promoter. EG103PZ cells in early to midlog phase were exposed either to PQ (1 mM or 3 mM PQ) or to a 38°C heat shock for 1 h. Both β-gal activity assays and β-gal ELISA were performed on the cells. However, only the β-gal ELISA results are shown, since both sets are concordant. The data (Fig. 4) reveals increased β-gal levels upon exposure to heat shock, but no stimulatory effect of PQ was demonstrated.

DISCUSSION

In this work, we have studied the effects of oxidative stress induced by paraquat on the yeast *PGK* promoter. This was done using a multicopy β-gal reporter plasmid, in which the *PGK* promoter, including the upstream activator sequence, was cloned in-frame with the *lacZ* gene of *E. coli*. This approach was chosen because it gives direct information regarding effects at promoter level. We carried out β-gal activity ONPG assays and β-gal ELISA, and found that results from the two methods were concordant. Moreover, the plasmid copy number was determined in cells exposed to PQ and was found to be similar to that in unexposed cells: 53 and 57 plasmids per cell, respectively (Fig. 2). This ruled out the possibility that our results were due to a selective effect of PQ on yeast cells synthesizing a higher amount of β-gal, because of harboring a greater number of β-gal reporter plasmids.

From the results, it has been shown for the first time that PQ, a $O_2^{\bullet-}$-generating compound, is able to stimulate transcription of the *PGK* promoter, thus confirming such indications from our previous work (3). In addition, we have observed that *PGK* promoter stimu-

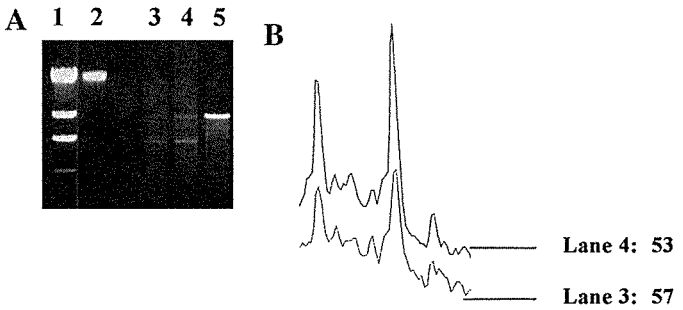

FIG. 2. Determination of plasmid copy number. An equal amount of total yeast DNA from cells grown in the presence and absence of 1 mM PQ, was digested with *Hin*dIII and run on an agarose gel as described under Materials and Methods (Fig. 2A). Lane 1: λ-DNA, cleaved with *Hin*dIII; lane 2: uncut total yeast DNA; lane 3: total yeast DNA from cells not exposed to PQ, digested with *Hin*dIII; lane 4: total yeast DNA from cells exposed to 1 mM PQ, digested with *Hin*dIII; lane 5: YEp363PZ linearized by *Hin*dIII. The upper band (in lanes 3 and 4) is the 9.4-kb fragment of linearized YEp363PZ plasmid; the lower band (in lanes 3 and 4) is the 6.4-kb repeat fragment from rDNA. In Fig. 2B, the profiles given by the bands in lanes 3 and 4 on scanning the gel with a fluorimager are shown, together with the calculated number of plasmids for each lane.

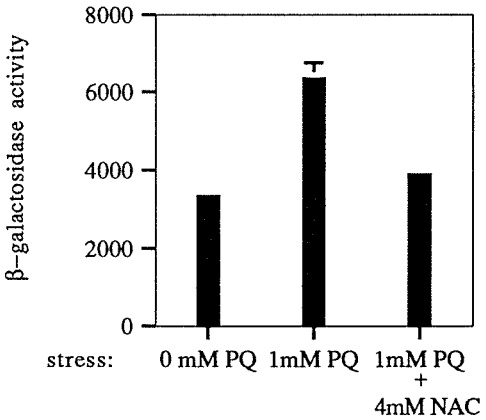

FIG. 3. Effect of *N*-acetylcysteine (NAC) on *PGK* promoter stimulation by paraquat. EG103PZ cells were grown on SC-Leu medium and treated with 1 mM PQ, or 1 mM PQ + 4 mM NAC, as described under Results. All values shown are the means of at least three independent determinations ± SD. Specific activity of β-gal is quoted as nmol ONPG hydrolyzed/min/mg total protein. Error bars are ±1 SD and appear where sufficiently large.

lation occurs (i) only when cells are exposed to sublethal, as opposed to lethal, concentrations of the oxidant (Fig. 1B), and (ii) only when cells are grown in the presence of PQ (and at midlog phase) as opposed to 1 h exposure to the oxidant (Figs. 1 and 4). Moreover, the antioxidant NAC (13) was found to attenuate *PGK* promoter stimulation by PQ (Fig. 3).

The most important conclusion from these experiments is the association between PQ exposure and stimulation of the *PGK* promoter. This is most likely to be the result of ROS production by PQ, as evidenced by the observed anti-stimulatory effect of NAC. A possible link between ROS generation and glycolytic stimulation is thus manifest, in other words, an adaptive stress response in which increased glycolysis enables the cell to meet falling energy production levels caused by oxidative stress, especially as a result of ROS mitochondrial damage. This is supported by the fact that PGK catalyses a step in glycolysis involving the production of ATP. Recent work linking ROS generation with *PGK* stimulation has shown that in wild-type hepatoma Hep3B cells ROS generation following incubation with cobalt chloride activates *PGK* mRNA, and that the antioxidants pyrrolidine dithiocarbamate and ebselen abolish transcriptional activation of the *PGK* gene (14).

In yeast, increased transcription of several glycolytic genes is known to occur in response to heat shock (15). Examples include glyceraldehyde-3-phosphate dehydrogenase (*HSP35*) (16) and enolase (*ENO1*) (17). As regards *PGK*, Piper *et al.* (18) have reported that PGK mRNA levels, even though already high in unstressed cells, are further enhanced within 30 to 60 min by a 25 to 38°C heat shock of *S. cerevisiae* growing on fermentative medium. This observation was confirmed in our experiments (Fig. 4). Piper *et al.* (18) concluded that the role of PGK may be to assist cells repressed in mitochondrial function during recovery following heat shock. In our experiments, the observation that *PGK* promoter stimulation was observed only at midlog phase in cells cultured in PQ-containing medium, and not after a 1 h exposure to the oxidant, further supports the notion that PGK is especially required in the recovery stage. Furthermore, it should be pointed out that both oxidative stress and heat shock are uncouplers of mitochondrial respiration (19, 20), and that heat shock may cause increased $O_2^{\bullet-}$ production (21). A

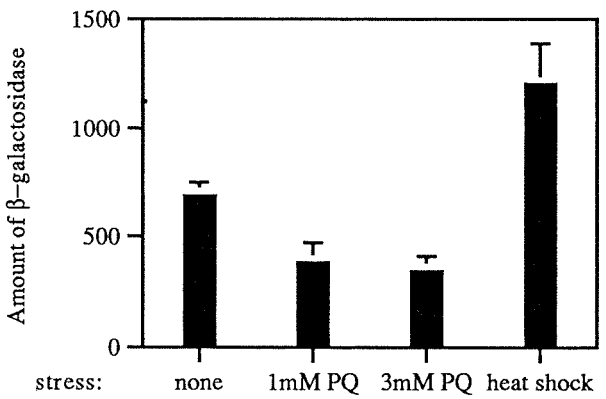

FIG. 4. Exposure of *S. cerevisiae* cells to paraquat and heat shock for 1 h. EG103PZ cells were treated with (i) 1 mM PQ, (ii) 3 mM PQ, and (iii) 38°C heat shock for 1 h. β-Galactosidase ELISA was carried out, as described under Materials and Methods. All values shown are the means of at least three independent determinations ± SD. Quantification of β-gal is quoted as ng of β-gal/mg total protein. Error bars are ±1 SD and appear where sufficiently large.

recent report by Yoo *et al.* (22) indicates that both PQ and heat shock activates the Cu,ZnSOD gene (*SOD1*) promoter through the same heat shock element (HSE). Since the *PGK* promoter also harbors a HSE, it would be interesting to analyze, using reporter gene deletion constructs, whether our observed ROS-induced *PGK* stimulation is being mediated via the HSE as well.

In summary, observations from this study have established a strong link between exposure of *S. cerevisiae* to the $O_2^{\bullet-}$-generating agent paraquat and stimulation of the glycolytic *PGK* gene promoter. This finding further implicates glycolysis as having an important role in the adaptive stress response of yeast cells, allowing cell growth in the face of moderately stressful environmental conditions. More work with other ROS-generating agents, such as menadione and plumbagin, is underway to further clarify these observations.

ACKNOWLEDGMENTS

We thank Alex Tzagoloff and David Stillman for supplying the plasmid YEp363 and Edith Gralla for strain EG103. N.V. is supported by a Research Grant from the Italo-Maltese Protocol Agreement (Project No. 15). D.R.G. is supported by a Research Fund Grant from the University of Malta.

REFERENCES

1. Santoro, N., and Thiele, D. J. (1997) Oxidative stress responses in the yeast *Saccharomyces cerevisiae, in* Yeast Stress Responses (Hohmann, S., and Mager, W. H., Eds.), pp. 171–211, Springer-Verlag, Heidelberg, Germany.
2. Hassan, H. M., and Fridovich, I. (1978) Superoxide radical and the oxygen enhancement of the toxicity of paraquat in *Escherichia coli. J. Biol. Chem.* **253,** 8143–8148.
3. Agius, D. R., Bannister, W. H., and Balzan, R. (1998) Prokaryotic iron superoxide dismutase replaces cytosolic copper, zinc superoxide dismutase in protecting yeast cells against oxidative stress. *Biochem. Mol. Biol. Int.* **44,** 41–49.
4. Stanway, C., Mellor, J., Ogden, J. E., Kingsman, A. J., and Kingsman, S. M. (1987) The UAS of the yeast *PGK* gene contains functionally distinct domains. *Nucleic Acids Res.* **15,** 6855–6873.
5. Winterbourn, C. (1993) Superoxide as an intracellular radical sink. *Free Radical Biol. Med.* **14,** 85–90.
6. Rose, M. D., Winston, F., and Hieter, P. (1990) Methods in Yeast Genetics: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Plainview, NY.
7. Myers, A. M., Tzagoloff, A., Kinney, D. M., and Lusty, C. J. (1986) Yeast shuttle and integrative vectors with multiple cloning sites suitable for construction of *lacZ* fusions. *Gene* **45,** 299–310.
8. Balzan, R., Bannister, W. H., Hunter, G. J., and Bannister, J. V. (1995) *Escherichia coli* iron superoxide dismutase targeted to the mitochondria of yeast cells protects the cells against oxidative stress. *Proc. Natl. Acad. Sci. USA* **92,** 4219–4223.
9. Sambrook, K. J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
10. Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
11. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* **153,** 163–168.
12. Broach, J. R. (1983) Construction of high copy yeast vectors using 2-microns circle sequences, *in* Methods in Enzymology (Wu, R., Grossman, L., and Moldave, K., Eds.), Vol. 101, pp. 322–325, Academic Press, NY.
13. Moldeus, P., Cotgreave, I. A. (1994) *N*-Acetylcysteine. *Methods Enzymol.* **234,** 482–492.
14. Chandel, N. S., Maltepe, E., Goldwasser, E., Mathieu, C. E., Simon, M. C., and Schumacker, P. T. (1998) Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. *Proc. Natl. Acad. Sci. USA* **95,** 11715–11720.
15. Mager, W. H., and Deknijff, A. J. J. (1995) Stress-induced transcriptional activation. *Microbiol. Rev.* **59,** 506–531.
16. Lindquist, S. (1986) The heat-shock response. *Annu. Rev. Biochem.* **55,** 1151–1191.
17. Iida, H., and Yahara, I. (1985) Yeast heat shock protein of M_r 48,000 is an isoprotein of enolase. *Nature* **315,** 688–690.
18. Piper, P. W., Curran, B., Davis, M. W., Lockheart, A., and Reid, G. (1986) Transcription of the phosphoglycerate kinase gene of *Saccharomyces cerevisiae* increases when fermentative cultures are stressed by heat-shock. *Eur. J. Biochem.* **161,** 525–531.
19. Lemasters, J. J., and Nieminen, A. L. (1997) Mitochondrial oxygen radical formation during reductive and oxidative stress to intact hepatocytes. *Biosci. Rep.* **17**(3), 281–291.
20. Schlesinger, M. J., Ryan, C., Chi, M. M., Carter, J. G., Pusateri, M. E., and Lowry, O. H. (1997) Metabolite changes associated with heat shocked avian fibroblast mitochondria. *Cell Stress Chaperones* **2,** 25–30.
21. Davidson, J. F., Whyte, B., Bissinger, P. H., and Schiestl, R. H. (1996) Oxidative stress is involved in heat-induced cell death in *Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA* **93,** 5116–5121.
22. Yoo, H. Y., Chang, M. S., and Rho, H. M. (1999) The activation of the rat copper/zinc superoxide dismutase gene by hydrogen peroxide through the hydrogen peroxide-responsive element and by paraquat and heat shock through the same heat shock element. *J. Biol. Chem.* **274,** 23887–23892.